Studies on Free and Enzyme-Bound Nicotinamide Adenine Dinucleotide Free Radicals

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Abstract: The spectral and kinetic properties of the NAD· free radical have been studied as a function of temperature and pH. The radical decays by second-order kinetics to an enzymatically inactive dimer $(NAD)_2$. At 23.5 °C and pH 7.3 the corresponding rate constant is $k_9 = (7.72 \pm 0.78) \times 10^7\ M^{-1}\ s^{-1}$ with an activation energy $E_a = 3.4 \pm 0.4$ kcal/mol. Upon attachment of the NAD· radical to an enzyme active site, the radical becomes stabilized. The stabilization effect (ratio of the rate of NAD· disappearance in the absence and presence of an enzyme) depends upon the nature of the enzyme and varies from 1.54×10^2 for alcohol dehydrogenase, 2.57×10^2 for malate dehydrogenase, 1.1×10^3 for lactate dehydrogenase, to 1.54×10^4 for glyceraldehyde-3-phosphate dehydrogenase. The observed second-order disappearance of enzyme-stabilized NAD· is explained by a mechanism that is dependent upon the dissociation constant of the enzyme–NAD· complex.

Introduction

Although it is generally believed that the enzymatically mediated oxidation of the reduced nicotinamide adenine dinucleotide (NADH) proceeds in a single step by a hydride ion (H^-) transfer,[2] other mechanisms taking into consideration two one-electron oxidation steps with the formation of a free radical (NAD·) intermediate have been reported.[3-6]

Methods by which this radical can be formed from a parent compound (either NADH or NAD^+) as well as studies of its chemical and kinetic properties were reported in a series of classical pulse radiolysis experiments by Land and Swallow[7,8] and Willson.[9] The radiation-induced reduction of NAD^+ to NAD· is most conveniently carried out in an oxygen-free formate solution according to the following scheme:[7]

$$H_2O \text{ —}\!\!\sim\!\!\rightarrow e_{aq}^-, H, OH, H_2O_2, H_2, H_3O^+ \quad (I)$$

$$OH + HCOO^- \rightarrow H_2O + CO_2^- \quad (1)$$

$$H + HCOO^- \rightarrow H_2 + CO_2^- \quad (2)$$

$$CO_2^- + NAD^+ \rightarrow CO_2 + NAD\cdot \quad (3)$$

$$e_{aq}^- + NAD^+ \rightarrow NAD\cdot \quad (4)$$

The radiation-induced oxidation of NADH to NAD· can be carried out in nitrous oxide saturated solutions containing an OH radical scavenger such as Br^-, I^-, or CNS^-.[8] Following reaction I:

$$e^- + N_2O + H^+ \rightarrow N_2 + OH \quad (5)$$

$$OH + Br^- \rightarrow Br\cdot + OH^- \quad (6)$$

$$Br\cdot + Br^- \rightarrow Br_2^-\cdot \quad (7)$$

$$Br_2^-\cdot + NADH \rightarrow NAD\cdot + 2Br^- + H^+ \quad (8)$$

The NAD· radical unlike other semiquinones does not disproportionate but preferentially dimerizes to an enzymatically inactive form:[8]

$$NAD\cdot + NAD\cdot \rightarrow (NAD)_2 \quad (9)$$

In the presence of O_2 as an electron acceptor, the radical NAD· is rapidly oxidized to enzymatically active NAD^+.[8,9]

$$NAD\cdot + O_2 \rightarrow NAD^+ + O_2^- \quad (10)$$

While neither NAD^+ nor NADH reacts with O_2^- at any appreciable rate,[8] NADH bound to an enzyme such as lactate dehydrogenase is rapidly oxidized in a chain reaction mechanism.[10-12]

$$\text{enzyme–NADH} + O_2^- \rightarrow \text{enzyme–NAD}\cdot + HO_2^- \quad (11)$$

$$\text{enzyme–NAD}\cdot + O_2 \rightarrow \text{enzyme–NAD}^+ + O_2^- \quad (12)$$

Attempts to reduce free or enzyme-bound NAD^+/NAD· to NADH by radiation have mostly been unsuccessful except in the work reported by Chan et al.[13] They have achieved reduction of $NADP^+$ to NADPH by radiation in the presence of ferredoxin–NADP reductase.

Earlier electron spin resonance (ESR) studies have shown that while well-resolved radical spectra could be obtained for NAD^+ analogues, the spectrum of NAD· itself was poorer in quality due to lack of intensity.[14]

The goal of the present investigation was to develop methods by which the NAD· radical either free or enzyme-bound could be stabilized for further studies and also to provide evidence for the existence of enzyme-bound NAD· radicals. We have compared the characteristics of the enzyme-bound and free nucleotide radicals under similar conditions. The spectral and kinetic data of the free NAD· radical in this study are in close agreement with earlier reports.[7,8]

Experimental Section

Materials. All solutions were prepared with highly purified water, which was obtained by passing distilled water through a Millipore ultrapurification system. Salts such as sodium phosphate and sodium formate were purified by recrystallization. A filtered source of UHP nitrogen (99.999%, Matheson Co.) was used to remove oxygen from solutions.

Yeast alcohol dehydrogenase (ADH) (EC 1.1.1.1), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12), bovine heart lactate dehydrogenase (LDH) (EC 1.1.1.27), and pig heart malate dehydrogenase (MDH) (EC 1.1.1.37) were obtained from Boehringer Mannheim Corp. The enzymes were dialyzed before use for 16 h at 4 °C against 0.05 M phosphate buffer at pH 7.3. Insoluble materials were removed by centrifugation, and the concentration of each enzyme was determined at 280 nm. The total number of active sites was evaluated by the method of Holbrook.[15]

Apparatus and Techniques. Pulse radiolysis experiments were carried out with a 2-MeV Van de Graaff accelerator. The sample cell had a 6.1-cm optical light path. All data were automatically evaluated on a PDP-11 computer. The energy input into the sample per pulse was evaluated from ferrous dosimetry calibrations using $G(Fe^{3+}) = 15.5$, where the G value represents the number of molecules formed or changed per 100 eV of energy dissipated.[16] All experiments were carried out at ambient temperature (23–24 °C).

Results

The NAD· Free Radical. The spectral and kinetic properties

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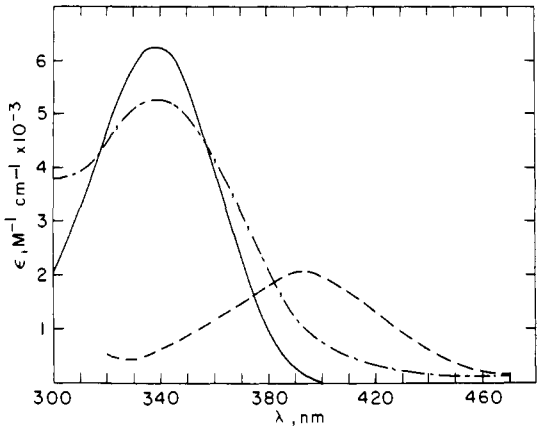

Figure 1. Absorption spectra of NADH (—), $(NAD)_2$ (- - -), and NAD· (· · ·) at pH 7.3 and 23.5 °C.

of NAD· were studied in a nitrogen-saturated 0.2 mM NAD^+ solution containing 0.1 M sodium formate. In this system the radical formation is described by reactions I and 1 through 4.

The absorption spectrum of NAD· shown in Figure 1 has a maximum at 390–395 nm with a molar absorbance of $\epsilon_{395nm}^{NAD\cdot} = (2040 \pm 20)\ M^{-1}\ cm^{-1}$. The latter was determined from a plot of optical absorbance as a function of energy-input/pulse, which is proportional to the radical concentration. The location of the absorption maximum and the magnitude of the molar extinction coefficient were found to be within experimental error independent of pH in the range between 5.5 and 9.2. Omission of sodium phosphate had no effect upon these spectral properties.

In the absence of appropriate reactants, the NAD· radical dimerizes according to reaction 9. This reaction follows strictly second-order kinetics and can be observed over several half-lives. The second-order rate constant which was computed by eq II,

$$k_9 = \left[\frac{\epsilon_{395nm}^{NAD\cdot} - {}^1/_2\epsilon_{395nm}^{(NAD)_2}}{2(t_2 - t_1)}\right][1/(OD)_{t_2} - 1/OD)_{t_1}] \quad \text{(II)}$$

$k_9 = (7.72 \pm 0.78) \times 10^7\ M^{-1}\ s^{-1}$ at 23.5 °C, was found to be within experimental error independent of pH in the range between 5.5 and 9.2.

Before determining the activation energy for reaction 9, it is necessary to establish if the molar absorbance of the NAD· radical is affected by temperature changes. Figure 2 shows that, while the absorbance of NAD· increases linearly with energy input (that is, with increasing radical concentration), it is not affected by the change in temperature over the range from 20 to 40 °C. These experiments were carried out at pH 7.3 and 395 nm.

The change in k_9 with temperature is given in Figure 3, where each point represents an average value of three runs. The activation energy computed from these data is $E_a = 3.4 \pm 0.4$ kcal/mol and $k_9 = 2.47 \times 10^{10} e^{-3400/RT}$ at pH 7.3.

$(NAD)_2$ Spectrum. The absorption spectrum of $(NAD)_2$ was determined with a Cary 14 spectrophotometer. The compound itself was prepared by ^{60}Co γ irradiation of a N_2-purged solution of 0.2 mM NAD^+ and 0.1 M HCOONa at pH 7.3 and 23.5 °C. The spectrum is shown in Figure 1. It has an absorption maximum at 345 nm with a corresponding molar absorption coefficient of $\epsilon = (5240 \pm 100)\ M^{-1}\ cm^{-1}$. While this absorption coefficient is in close agreement with a recently reported value for $(NADP)_2$ by Chan et al.,[13] it is significantly lower than the corresponding values reported by Burnett and Underwood[17] and Land and Swallow[7] by 16 and 22%, respectively.

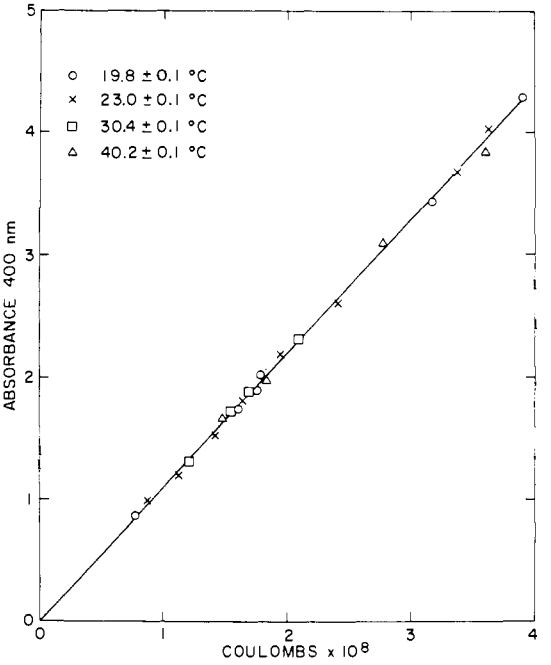

Figure 2. Absorbance of NAD· at 395 nm and pH 7.3 as a function of energy input per pulse. Measurements taken at the different temperatures show that the absorbance of NAD· is temperature independent.

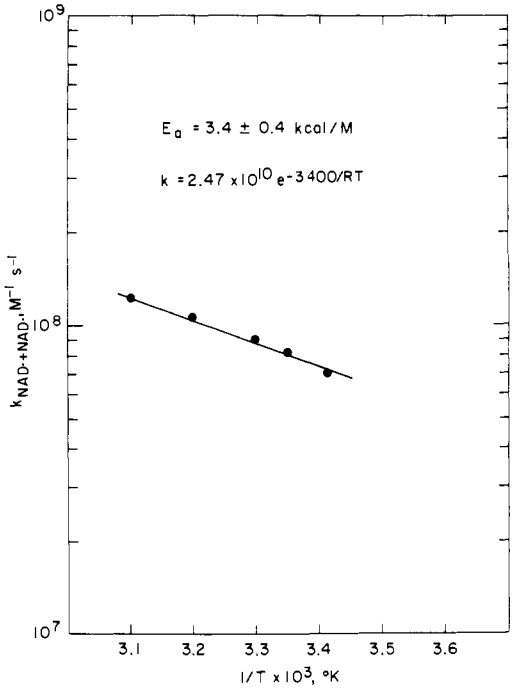

Figure 3. k_9 as a function of temperature. The rate constants were computed from measurements taken at 395 nm in N_2(UHP)-purged solutions containing 0.1 M formate, 0.2 mM NAD^+, and 4 mM phosphate at pH 7.3.

Addition of an equal molar amount of either GAPDH or MDH to a $(NAD)_2$ solution at pH 7.3 had no effect on the $(NAD)_2$ absorption spectrum.

Enzyme–NAD· Radical Complex. The NAD· radical can be significantly stabilized when attached to the active site of an appropriate enzyme. In the present investigation the free-radical NAD· was formed by reduction of NAD^+ with CO_2^- as described by reactions 1–4 in the presence of an enzyme. Since both free and bound NAD· react with O_2 at near diffusion-controlled rates (reactions 10 and 12), the success of such a study depends very much on how completely molecular oxygen is removed from the given enzyme-formate-NAD^+

Table I. Rate and Dissociation Constants for Dehydrogenase and NAD·[a]

enzyme	k_{-14}, M^{-1} s^{-1}	k_{13}, M^{-1} s^{-1}	$K_{E\text{-}NAD\cdot}$, M	$K_{E\text{-}NADH}$, M	k_9/k_{13}
ADH	5.0×10^7	$(5.0 \pm 0.8) \times 10^5$	2.4×10^{-6}	$(10^{-4}$–$10^{-5})^b$	154
MDH	1.2×10^8	$(3.0 \pm 0.4) \times 10^5$	1.5×10^{-6}	$(10^{-5}$–$10^{-6})^c$	257
LDH	1.0×10^9	$(7.0 \pm 1.2) \times 10^4$	7.6×10^{-7}	$(3.9 \times 10^{-7})^d$	1 100
GAPDH	2.4×10^9	$(5.0 \pm 1.0) \times 10^3$	4.0×10^{-7}	$(10^{-9})^e$	15 400

[a] Measurements were taken in solutions containing 100–200 μM NAD^+, 15–80 μM active enzyme sites, 1–2 μM NAD·, and 0.05 M phosphate. We are assuming that under these conditions we are dealing with K_1s only. The dissociation constants ($K_{E\text{-}NADH}$) for ADH, MDH, and LDH are average values for all active sites of the given enzyme. [b] References 19 and 20. [c] References 21 and 22. [d] Reference 23. [e] Reference 24.

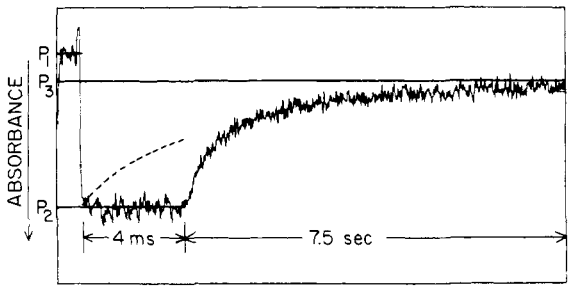

Figure 4. Experimental run showing change in absorbance with time (note split time base) at 410 nm. The sample contained 1 M formate, 0.05 M phosphate, 0.25 mM NAD^+, and 30 μM malate dehydrogenase active sites, pH 7.3 at 24 °C. P_1 = absorbance before irradiation with a 10-μs electron pulse; P_2 = absorbance due to MDH-NAD·; P_3 = absorbance due to product $(NAD)_2$ at infinite time. Drawn-in broken line shows the decay of an identical quantity of NAD· (control experiment) in the absence of enzyme.

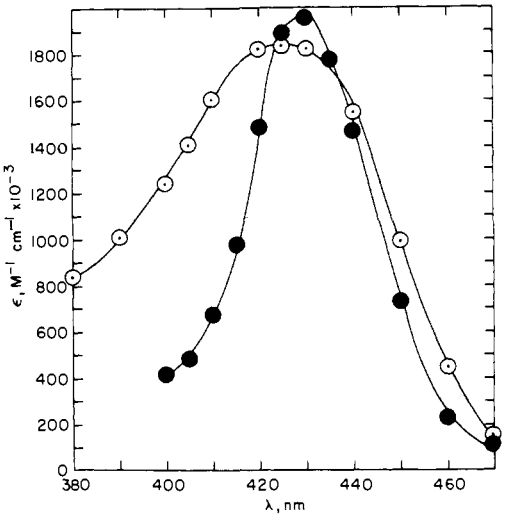

Figure 5. Absorption spectra of MDH-NAD· (○) and GAPDH-NAD· (●) in 1 M HCOONa, phosphate, 0.05 M at pH 7.3 and 24 °C.

solution. We have encountered great difficulties in lowering the molecular oxygen concentration below micromolar level. The higher the molecular weight of the enzyme, the harder it is to remove the last traces of O_2 due to extensive foaming and denaturation of the enzyme. Consequently in this study residual O_2 was removed by converting it to O_2^- by irradiation. On the average, 4–5 pulses (1 krad/pulse) were sufficient to lower oxygen concentration to a negligible level. The method was found to be satisfactory since the high concentration of formate could protect the enzyme from radiation damage while the CO_2^-, NAD·, and enzyme-NAD· converted O_2 to O_2^-. The resulting O_2^- decays to O_2 and H_2O_2. Thus, if a N_2-purged solution contained 1 μM O_2, five electron pulses would reduce the oxygen content to approximately 0.06 μM.

Following the initial prepulsing for removal of oxygen, the observed initial absorbance due to NAD· or enzyme-NAD· was constant for each pulse up to 15 consecutive pulses when the energy input per pulse was kept constant. In this study a sample was pulsed 10–12 times.

The MDH-NAD· complex was studied most extensively because solutions of this enzyme were the easiest to deoxygenate. A typical run is illustrated in Figure 4 where P_1 = absorbance before the electron pulse, P_2 = total absorbance due to MDH-NAD· during the first 4 ms following the electron pulse, and P_3 = computed absorbance due to $(NAD)_2$ at infinite time. As indicated, this experiment was carried out with a split time base. There is no decay of MDH-NAD· during the first 4 ms. The drawn-in broken line illustrates a control run in which the same amount of NAD· free radical decays spontaneously in the absence of the enzyme. Upon switching to a 1000-fold longer time scale, the decay of MDH-NAD· can be observed over a time period of approximately 7.5 s.

The absorption spectrum of MDH-NAD· in Figure 5 was determined by plotting (P_2 P_1) as a function of wavelength. The molar extinction coefficient of MDH-NAD· was determined at 425 nm from a plot of $\Delta(P_2 - P_1)$ vs. energy input per pulse (data not shown). The molar extinctions at other wavelengths are normalized values based upon the 425-nm measurements.

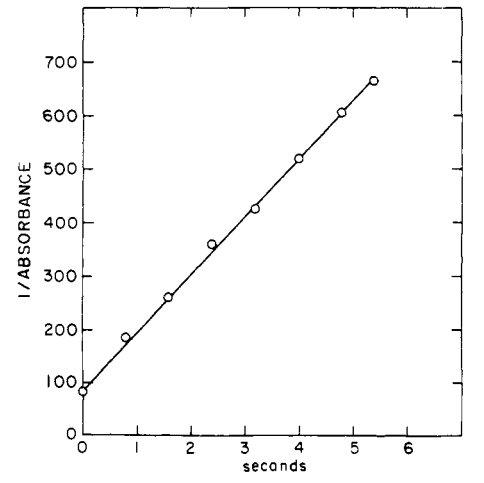

Figure 6. Reciprocal absorbance change at 430 nm as a function of time. Light path 6.1 cm, pH 7.3, temperature 24 °C. The decrease in absorbance is due to the second-order disappearance of MDH-NAD·. The rate for this particular experiment is $k_{13} = 2.6 \times 10^5$ M^{-1} s^{-1}. The solution contained 1.0 M sodium formate, 0.05 M phosphate, 0.25 mM NAD^+, and 30 μM malate dehydrogenase active sites.

The MDH-NAD· radical complex decays by second-order kinetics as illustrated in Figure 6 and as described by the overall reaction 13:

$$\text{MDH-NAD}\cdot + \text{MDH-NAD}\cdot \rightarrow 2\text{MDH} + (\text{NAD})_2 \quad (13)$$

The corresponding rate constant is $k_{13} = 3.0 \times 10^5$ M^{-1} s^{-1}.

Similar studies were carried out to determine the second-order rate constants for the decay of the other enzyme-NAD· radicals. They were computed from eq II by substituting $\epsilon^{\text{enzyme-NAD}\cdot}$ for $\epsilon^{\text{NAD}\cdot}$. The results are listed in Table I. The

corresponding effective extinctions per active-site NAD· of all the enzymes studied were found to be similar (within 15–20%) in the wavelength region between 420 and 440 nm.

Discussion

As indicated from the activation energy, lowering of temperature does not significantly decrease the rate of reaction 9 to be of practical use (by a factor of 20/100 °C). Significant stabilization of the NAD· radical, however, can be achieved by its attachment to an appropriate enzyme site (Table I). The following observations support the fact that the NAD· radical is bound to the active site of the enzymes: (a) It has been shown in earlier work that a NAD· radical can be formed at the LDH active site when NADH is oxidized in two single steps to NAD^+ by malate free radicals. The transfer of the hydrogen atom is stereospecific.[18] (b) The dissociation constants for the attachment of NAD· to the first active site of a given enzyme listed in Table I follow the same overall trend as the corresponding dissociation constants for enzyme-NADH.[19-24] (c) Protein molecules like ovalbumin have no stabilizing effect upon NAD· free radicals.

The rate and dissociation constants listed in Table I show that the stabilizing effect (k_9/k_{13}) depends upon the nature of the enzyme and varies from 150 for ADH to 1.5×10^4 for GAPDH.

Upon attachment to the enzyme the spectrum of NAD· undergoes a red shift of about 30–35 nm. This can be seen by comparing the two spectra for bound NAD· in Figure 5 with that of free NAD· in Figure 1. It is reasonable to assume that the observed spectral shift occurs because in aqueous solutions the intermediate free-radical NAD· is present in a folded conformation, while when attached to the enzyme it is unfolded. That such conformational changes result in spectral shifts had been shown by extensive studies of NAD^+/NADH and derivatives by fluorescence emission, proton magnetic resonance, and circular dichroism.[25-30]

The enzyme-NAD· spectra for ADH and LDH are similar to that of MDH (data not shown), but the spectrum of GAPDH-NAD· has a sharper and less diffused profile (Figure 5). It is possible that the difference in the spectra between GAPDH-NAD· and the other three enzyme-NAD· complexes (ADH-NAD·, LDH-NAD·, and MDH-NAD·) is due to the difference in the three-dimensional structure of the active sites. Rossmann et al.[31] have found that the nucleotide binding cavity of GAPDH is less hydrophobic than those of MDH, LDH, and ADH. The three-dimensional orientation of bound NAD^+ is similar for MDH, LDH, and ADH, while the pyridine ring of the GAPDH-bound nucleotide has a 180° rotation about the glycosidic bond linking the pyridine ring to the ribose. Consequently, in LDH, MDH, and ADH the C-4 hydrogen on the A side of the bound NADH is transferred to a substrate, in contrast to GAPDH in which the transferable hydrogen is on the B side of the pyridine ring.

In a nucleotide analogue study, a similar spectral shift was reported earlier by Kosower et al.[32] They observed that upon acidification of the 1-methyl-4-carbamidopyridinyl radical the spectrum shifted from 405 to 425 nm and suggested that it may be due to protonation of the amide group. Further study is needed to determine if a similar situation prevails at the enzyme binding site.

Since there is no significant change in the molar absorbance nor in the overall profile of the spectrum, it may be assumed that the NAD· radical when attached to the enzyme retains its integrity and that there is little interaction between the unpaired electron of the free radical and the enzyme.

Under present experimental conditions in which [NAD^+] > [active sites], the NAD· was preferentially generated as an unbound radical which subsequently diffused to an active site. In order to account for the second-order disappearance of E-NAD· (reaction 13) and the fact that the only reaction product observed and quantitatively accounted for is $(NAD)_2$, one has to assume a mechanism which involves a rapid equilibrium between free and enzyme-bound NAD· in addition to the dimerization reaction (i.e., reaction 13 is a composite of reactions 14 and 9):

$$\text{E-NAD}\cdot \rightleftharpoons \text{E} + \text{NAD}\cdot \qquad (14,-14)$$

$$\text{NAD}\cdot + \text{NAD}\cdot \rightarrow (\text{NAD})_2 \qquad (9)$$

$$K_{\text{E-NAD}\cdot} = \frac{[\text{enzyme}][\text{NAD}\cdot]}{[\text{enzyme-NAD}\cdot]} \qquad (III)$$

If the total number of radicals is [R·] = [NAD·] + [E-NAD·], it can be shown that:

$$\frac{d[R\cdot]}{dt} = \frac{2k_9[R\cdot]^2}{[1 + [E]/K]^2} \qquad (IV)$$

where [E] is the concentration of active enzyme sites and K is given by eq III. As is apparent, eq IV predicts a second-order decay consistent with the experimental observations (Figure 6).

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